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14. ABSTRACT

Our purpose is to examine the role of the NF1 gene product, neurofibromin, in modulating the migratory and invasive properties of neural crest cells (NCC) and neural crest-derived sarcoma cells. As a negative regulator of Ras signaling, neurofibromin may influence the responses of NC-derived cells to growth factors and extracellular matrix (ECM) molecules that affect motility. We have completed our analyses of Nf1-/- embryonic NCC invasiveness *in vitro*, and compared effects of neurofibromin deficiency in different embryonic mesenchymal cell populations derived from cranial and trunk regions. We have used immunoblotting techniques to characterize signaling pathways activated by TGF-beta and PDGF-BB in MPNST-like sarcoma cell lines isolated from cisNf1+/-;p53+/- mice, and compared effects of these growth factors on invasiveness through laminin. Finally, we have expanded our analyses of the cisNf1+/-;p53+/- mouse model to include characterizations of genomic instability in the context of malignant transformation, and to test possible modifiers of MPNST growth and invasiveness.

15. SUBJECT TERMS
neurofibromatosis type 1; neural crest cells; cell motility and Migration; PDGF; TGF-beta; MPNST, neurofibromin, genomic instability

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INTRODUCTION

For a number of normal and neoplastic cell types, loss or diminution of the Nf1 gene product, neurofibromin, leads to changes in migratory behavior, in particular invasiveness. These alterations in cell motility potentially affect the establishment and growth of neurofibromas and café-au-lait macules, metastasis of malignant peripheral nerve sheath tumors (MPNST), and the development of subtle central nervous system abnormalities that could contribute to learning disorders in NF1 patients. The main objectives are to characterize the changes in cell motility for neurofibromin-deficient embryonic neural crest cells, and to identify environmental cues that influence the invasiveness of MPNST cell lines derived from spontaneous tumors in *cisNf1+/-;p53+/-* mice. During the past two years, we completed a project to compare *Nf1*+/+, +/-, and -/- neural crest cell migration through fibronectin and laminin matrices, and identified platelet-derived growth factor (PDGF) and PDGF receptor signaling pathways that influence proliferation and migration of MPNST cell lines. These data allowed us to obtain additional funding from university sources, and thus we have included two projects that were not in the original Statement of Work, but which were outgrowths of the proposal.

BODY

Neural Crest Cell Invasiveness (revised SOW, Task 2)

Several different classes of signaling molecules, distributed along migration routes and within localization sites, influence the motility, proliferation, survival, and differentiation of neural crest-derived cells throughout development. Many of these environmental cues directly or indirectly affect early aspects of NCC behavior by signaling through receptor tyrosine kinases. Neurofibromin, a GAP encoded by the neurofibromatosis type 1 (Nf1) gene, has a crucial role as a negative regulator of RTK signaling through Ras in neural crest-derived cells. Mouse embryos that lack neurofibromin (*Nf1-/-*) die before embryonic day 14 (E14: Brannan et al., 1994; Jacks et al., 1994); however, many neural crest-derived cell types can be isolated prior to this stage and maintained in culture. Sensory and sympathetic neurons isolated from Nf1-deficient mouse embryos survive and extend neuritis in the absence of neurotrophins required by wild-type cells, presumably due to constitutive Ras activation (Vogel et al., 1995, 2000). Loss of neurofibromin in Schwann cells leads to accelerated differentiation (Kim et al., 1995), unless hyperproliferation is induced by increasing cyclic AMP levels (Kim et al., 2001). To characterize the effects of *Nf1* gene dosage on the motility of neural crest-derived cells, we isolated first branchial arch mesenchymal populations, as well as trigeminal ganglion non-neuronal cells, from mouse embryos and measured their performance in transwell invasiveness assays. In agreement with results reported for other cell types, we find that neurofibromin deficiency significantly increases the invasive potential of cranial neural crest populations *in vitro*.

Figure 1 summarizes the results of experiments that demonstrate that loss of neurofibromin affects the invasiveness of neural crest-derived (trigeminal ganglion) and cranial mesenchymal (branchial arch) cell populations, but not the invasiveness of trunk mesodermal (limb) cells at the stages examined. In addition, a comparison of the invasiveness of Nf1-/- trigeminal and branchial arch cells between E10 and E12 indicates that the roles of neurofibromin in controlling motility may become increasingly important as development proceeds. **Figure 2** shows that the PI3-kinase inhibitor, LY294002, attenuates the increased migration of *Nf1-/-* trigeminal neural crest cells; we have also decreased invasive capacity by treating these cells with the MAP kinase inhibitors PD98059 and U0126 (data not shown). These data are consistent with results using neurofibromin-deficient Schwann cells derived from E12.5 dorsal root ganglia (Huang et al., 2004), and are combined with our earlier results in a manuscript submitted for review, to the journal *Developmental Dynamics* (July 2005).

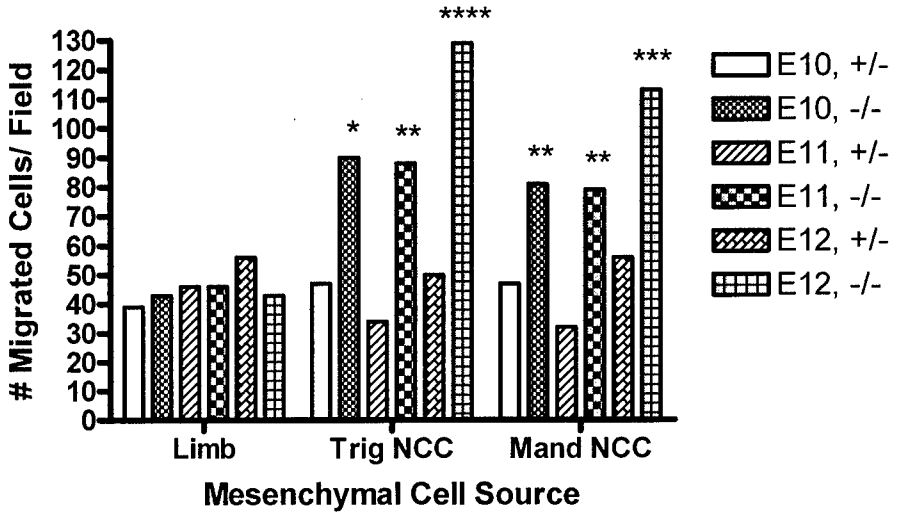
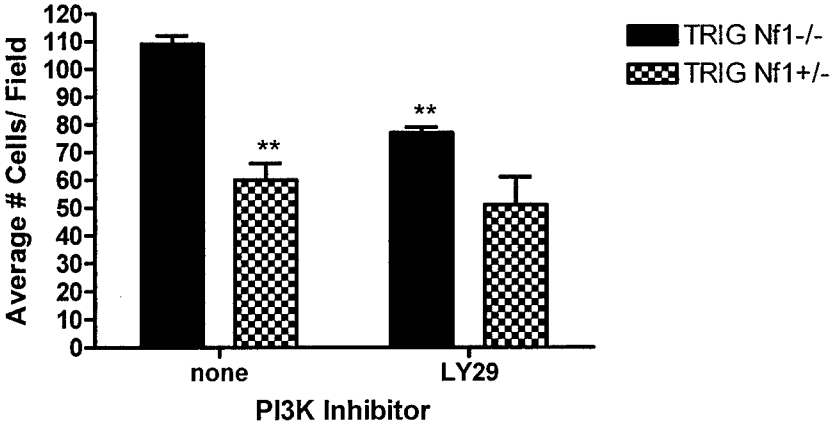


Figure 1. Invasiveness of embryonic mouse mesenchymal cells through laminin. For each bar, 4-8 independent transwells were scored. * $p<0.001$, ** $p<0.0001$, *** $p<0.00001$, **** $p<0.000001$

Figure 2.
Effect of PI3 Kinase Inhibitor on
Migration of E13 Trigeminal NCC



TGF-β and PDGF Signaling in MPNST Cell Lines (revised SOW Tasks 3 and 4)

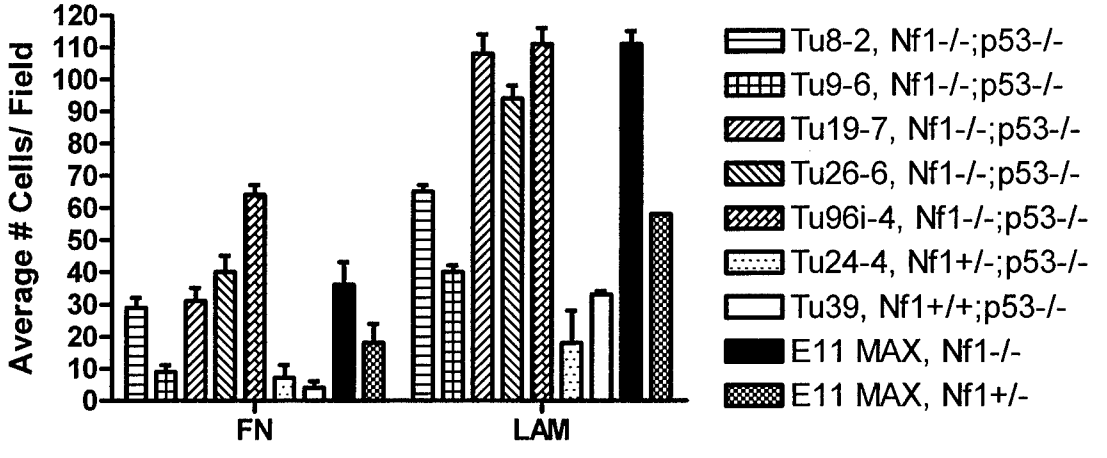
MPNST consist primarily of Schwann cells, a glial cell type derived from the neural crest. As neural crest cells differentiate into Schwann cells, they acquire responsiveness to an increasing number of growth factors that can block cell death (Woodhoo et al., 2004). For example, fibroblast growth factor-2 (FGF-2) and endothelins can rescue early Schwann cell precursors, but not undifferentiated neural crest cells. Dorsal root ganglion-derived satellite cells, which are closely related to Schwann cells, exhibit an accelerated development of responsiveness to survival factors such as platelet-derived growth factor-BB (PDGF-BB; Woodhoo et al., 2004). Other growth factors, in particular transforming growth factor-beta (TGF-β), elicit Schwann cell death (Parkinson et al., 2001).

Cell lines derived from soft tissue sarcomas in *cisNf1+/-;p53+/-* mice express different combinations of neural crest cell markers (Vogel et al., 1999). Like normal Schwann cells and satellite cells, most of our

MPNST lines express S100β, p75 neurotrophin receptor, neural cell adhesion molecule, and glial fibrillary acidic protein; in addition, some lines express markers characteristic of smooth muscle and neuronal differentiation. MPNST cell lines also differ in their invasiveness through matrices of fibronectin and laminin (**Figure 3**), which are extracellular proteins that influence normal neural crest cell migration during development.

To begin to identify changes in growth factor responsiveness that may lead to malignant transformation of plexiform neurofibromas, and may account for differences in proliferative capacity and invasiveness between our neural crest-derived sarcoma lines, we have examined signaling pathways activated by TGF-β and PDGF-BB. **Figure 4** (see page 10) summarizes results of Western blot experiments using antibodies against activated forms of Akt (PI3 kinase pathways), and Erk1 and 2 (MAP kinase pathway), for three MPNST cell lines following TGF-β treatment. Line Tu26-6, which has a Schwann cell phenotype and is highly invasive through laminin, does not respond to TGF-β treatment with diminished invasiveness or with changes in proliferation. **Figure 5** (see page 11) summarizes results of Western blot experiments for a number of different MPNST lines, following exposure to PDGF-BB. Again, there is heterogeneity in responsiveness to PDGF-BB among our different MPNST lines, particularly in the activation of the PDGF receptor; we have also found that production of the PDGF ligand varies between cell lines, raising the possibility that some MPNST may exhibit autocrine stimulation of PDGF signaling pathways.

Figure 3.
Migration of Sarcoma Cells and NCC through Fibronectin and Laminin



Recently, Dang and DeVries (2005) reported abnormal responses to PDGF-BB in human MPNST, as compared to normal Schwann cells. We will complete our analyses of PDGF-BB effects on mouse sarcoma cell invasiveness and signal transduction within the next few months; in addition, we will include experiments to examine calcium-mediated signaling, as reported by Dang and DeVries (2005) for human MPNST. These PDGF-BB results will be combined for a manuscript; the TGF-β signaling results will be combined with data on Jun kinase activation and apoptosis induction for a separate manuscript.

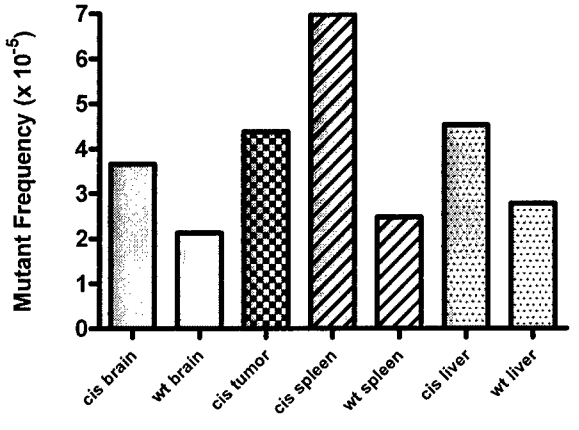
Spontaneous Mutant Frequency in Mouse MPNST

Because both the *Nf1* and *p53* gene products suppress proliferation and promote apoptosis in neural crest-derived cells, and because loss-of-heterozygosity at both loci occurs in the *cisNf1+/-;p53+/-* sarcomas, we wanted to determine whether a mutator phenotype arises in the murine MPNST. To begin to address this question, we combined the *cisNf1+/-;p53+/-* mice with Stratagene Big Blue *lacI* transgenic mice, so that we could quantitate spontaneous mutant frequencies in tumors and normal tissues isolated from the compound heterozygotes, and compare these values to those obtained from wild-type, age-matched littermates. To characterize the tumor spectrum in the presence of the *lacI* transgene, and to

ensure that it included MPNST as reported earlier (Vogel et al., 1999), we performed immunohistochemistry on tumor sections, using antibodies that recognized markers for Schwann cell (S100, CNPase), smooth muscle (smooth muscle actin, calponin), and neural (peripherin) differentiation. Twenty-three of the 27 tumors examined expressed S100 protein in the majority of cells; in addition, 12/27 tumors expressed significant levels of a smooth muscle marker protein, calponin. As reported previously (Vogel et al., 1999), we often observed expression of several different neural crest lineage markers within the same tumor.

CisNf1+/-;*p53*+/-;*lacI*+ mice were housed with wild-type littermates, and monitored twice each week for tumor formation and signs of illness, lethargy, and ataxia. When illness or tumor size and location interfered with normal behavior and feeding, the mouse was euthanized and tissues (tumor, brain, liver, spleen) were harvested and flash-frozen for isolation of genomic DNA and *lacI* mutant frequency assays. Whenever possible, brain, spleen, and liver were isolated from a wild-type *lacI*+ littermate at the same time, for direct comparison in mutant frequency (MF) assays. **Figure 6** provides a summary of average MF values for all *cisNf1*+/-;*p53*+/- and wild-type tissues examined. For *cisNf1*+/-;*p53*+/- mice, at least 11 of the 41 tumor MF, and 8 of the 35 brain MF, values were above the highest value obtained for wild-type brain. We obtained spontaneous mutant frequency values for the source of MPNSTs, peripheral nerve, by pooling tissues (sciatic nerve and trigeminal ganglia) dissected from several mice of the same genotype, at approximately the same age (5-6 months). In two independent experiments for both *cisNf1*+/-;*p53*+/- and wild-type mice, we obtained MF values of $2.3 \times 10^{-5}/2.4 \times 10^{-5}$ and $0.67 \times 10^{-5}/3.0 \times 10^{-5}$, respectively. Both neural crest-derived sarcomas and brains isolated from *cisNf1*+/-;*p53*+/- mice displayed heterogeneity in MF, with some values exhibiting a 2.8- to 5.4-fold increase when compared to the average for wild-type brain MF. Moreover, many of the tumors had higher mutant frequencies than those observed for grossly normal peripheral nerve in either *cisNf1*+/-;*p53*+/- or wild-type mice.

Figure 6.
Summary of Mutant Frequency Data
cisNf1+/-;p53+/- vs. Wild-type



To determine whether *Nf1* and *p53* deficiencies affected spontaneous mutagenesis in non-neural tissues, we compared *lacI* transgene mutant frequencies in spleens and livers isolated from *cisNf1*+/-;*p53*+/- and wild-type mice. Of the 17 *cisNf1*+/-;*p53*+/- spleens with elevated MF (above wild-type average), 8 were abnormal in size or gross appearance upon necropsy. However, several *cisNf1*+/-;*p53*+/- spleens were enlarged, yet had mutant frequencies well within the normal wild-type range, and the spleen with the highest MF (2.55×10^{-4}) was normal in gross appearance. Similarly, Giese and colleagues (2002) failed to observe a correlation between size abnormalities and increased MF in the spleens of *p53*-/-;*lacZ* mice. Thus, both spleens and livers isolated from *cisNf1*+/-;*p53*+/- mice displayed increases in MF, indicating that spontaneous mutagenesis may be elevated in tissues not typically associated with NF1 tumors. Our mouse MPNST model, which we characterized using DoD funding, and our data on the

heterogeneity of MPNST cell line invasiveness and growth factor responsiveness, allowed us to obtain independent funding for the mutant frequency project. We expect that continued investigations of genomic instability, in the context of MPNST and NF1 mouse models, will be a major focus of our research program in the future.

Mrg15 as a Modifier of MPNST Growth

To begin to identify possible modifiers of the malignant NF1-associated tumor phenotype, we combined our cis*Nf1;p53* mutations with a targeted null mutation in the *Mrg15* gene. *Mrg15* encodes a chromodomain protein that is present in complexes involved in transcriptional activation (Pardo et al., 2002). *Mrg15*-/- mouse embryos typically die around E14.5, and exhibit defects in the forebrain, heart, and lungs; in addition, *Mrg15*-/- mouse embryonic fibroblasts exhibit a proliferation defect (K. Tominaga, pers. comm.). Because MRG15 protein derepresses the *B-myb* promoter by association with retinoblastoma protein, thus promoting cell cycle progression, we reasoned that a reduction in Mrg15 might affect tumor latency and cell proliferation characteristics in *cisNf1*+/-;*p53*+/- mice. **Table 1** summarizes our preliminary data, and indicates that although heterozygosity for Mrg15 may not affect tumor latency, it may slow the tumor growth rate.

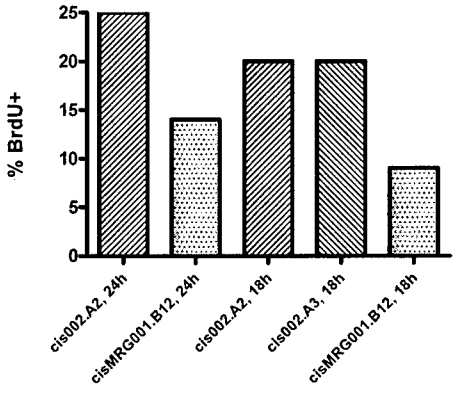
Table 1. Testing Mrg15 as a Possible Modifier of cisNf1+/-;p53+/- Tumor Latency and Growth

Mouse ID	Genotype	Age (mos.)	Tumor/Pathology	Tumor Growth Rate
cisMRG2-3#57	cisNf1+/-;p53+/-Mrg15+/-	3.0	Thoracic mass (1.8x1.8x1.2cm)	Unknown
cisMRG1-2#15	cisNf1+/-;p53+/-Mrg15+/+	4.3	Tumor in abdominal wall (1.2x1.0x0.4cm)	Unknown
cisMRG1-1#8	cisNf1+/-;p53+/-Mrg15+/-	4.5	Tumor ventral hindlimb (3.1x2.4x1.3cm)	Moderate
cisMRG1-1#6	cisNf1+/-;p53+/-Mrg15+/+	4.7	Thoracic mass (1.5x1.5x0.5cm)	Unknown
cisMRG1-1#5	cisNf1+/-;p53+/-Mrg15+/-	4.7	Thoracic mass (1.8x1.8x0.7cm)	Unknown
cisMRG1-2#14	cisNf1+/-;p53+/-Mrg15+/+	4.7	Ataxia, paralysis, cerebellar tumor?	Unknown
cisMRG4-1#38	cisNf1+/-;p53+/-Mrg15+/-	4.7	Forelimb tumors (1.8x1.6x0.6cm and 1.6x1.7x0.8cm)	Slow
cisMRG2-3#63	cisNf1+/-;p53+/-Mrg15+/+	5.0	Ataxia, brain tumor?	Unknown
cisMRG2-3#64	cisNf1+/-;p53+/-Mrg15+/-	5.7	Forelimb tumor (1.4x1.5x1.1cm)	Slow
cisMRG2-2#49	cisNf1+/-;p53+/-Mrg15+/-	6.0	Possible thyroid tumor (2x1.5x1.4cm)	Moderate
cisMRG2-3#58	cisNf1+/-;p53+/-;Mrg15+/-	6.0	Nerve-associated tumor, hindleg (3.2x2.9x2.7 cm)	Moderate
cisMRG1-2#13	cisNf1+/-;p53+/-Mrg15+/+	6.3	Thoracic mass (2x1.6x0.8cm)	Unknown
cisMRG2-1#41	cisNf1+/-;p53+/-Mrg15+/+	7.3	Brain hemorrhage, brain tumor?	Unknown
cisMRG2-1#40	cisNf1+/-;p53+/-Mrg15+/-	7.3	Tumor on flank (0.4x0.6x0.6 cm); perineal tumor (?)	Very slow
cisMRG2-2#56	cisNf1+/-;p53+/-Mrg15+/-	7.5	Unknown; no external tumor	N/A
cisMRG1-2#21	cisNf1+/-;p53+/-Mrg15+/+	8.0	Hindlimb and flank tumors (1.5x1.4x1.1, 1.5x1.3x0.6)	Moderate, Rapid

cisMRG1-2#20	cisNf1+/-;p53+/-Mrg15+/-	8.0	Small tumor, nasal cavity	Unknown
cisMRG1-1#7	cisNf1+/-;p53+/-Mrg15+/+	8.3	Perineal tumor (3.5x1.4x2.2cm)	Rapid
cisMRG1-1#10	cisNf1+/-;p53+/-Mrg15+/+	8.3	Brain tumor; abdominal wall tumor	Unknown

We have begun to characterize clonal cell lines isolated from these tumors, for both proliferative capacity *in vitro*, and alterations in *Mrg15* gene dosage. The results of preliminary bromodeoxyuridine (BrdU) labeling studies are presented in **Figure 7**. MPNST cell lines derived from tumors arising in Mrg15+/- mice appear to have reduced proliferative capacity, when compared to those isolated from mice that have two intact copies of *Mrg15*. Again, our mouse MPNST motility and growth factor responsiveness data allowed us to obtain additional independent funding, in order to characterize *Mrg15* as a potential modifier of tumorigenesis in the context of NF1.

Figure 7.
BrdU-Labeling of Cells Derived from cisNf1;p53 and cisNf1;p53;Mrg15 Tumors



cis002.A2, A3: derived from cisNf1+/-;p53+/-; Mrg15+/+ mouse
cisMRG001.B12: derived from cisNf1+/-;p53+/-;Mrg15+/- mouse

Figure 4A.
Comparison of Phospho-Akt1 Levels
in Cell Lines Grown in TGF-Beta (5 ng/ml)
1-24-05

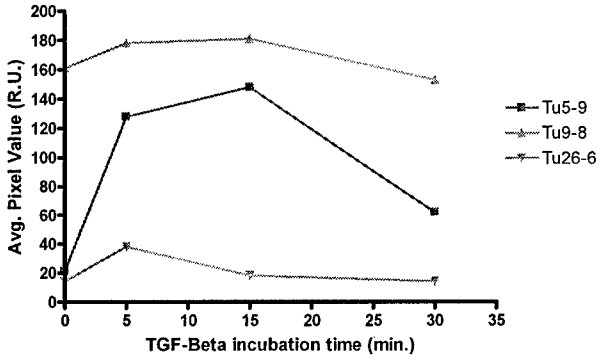


Figure 4B.
Comparison of Phospho-Erk1 Levels
in Cell Lines Grown in TGF-Beta (5 ng/ml)
1-24-05

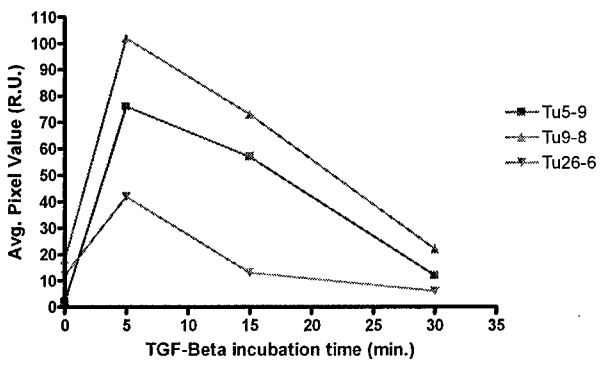


Figure 4C.
Comparison of Phospho-Erk2 Levels
in Cell Lines Grown in TGF-Beta (5 ng/ml)
1-24-05

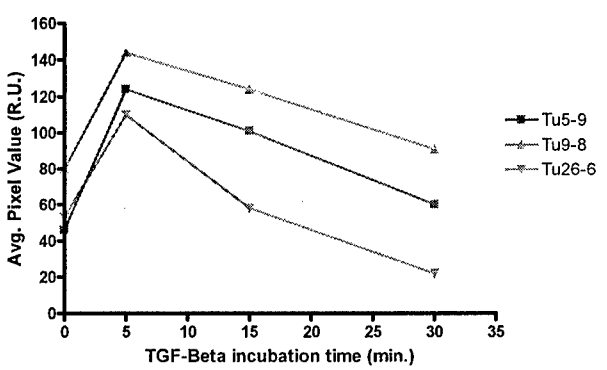


Figure 5A.
Comparison of Phospho-PDGF Receptor Beta Levels in Tumor Samples Grown in PDGF (10 ng/ml), 12-9-04

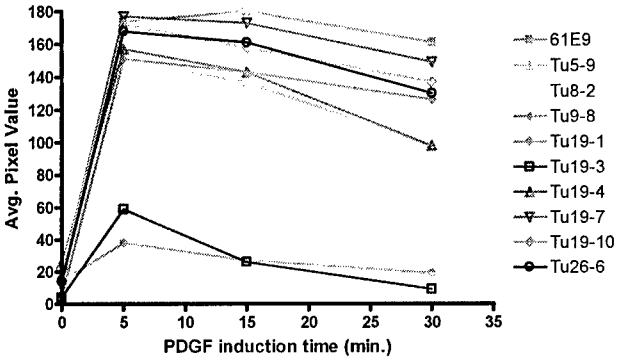


Figure 5B.
Comparison of Phospho-Akt1 Levels in Tumor Samples Grown in PDGF (10 ng/ml), 12-9-04

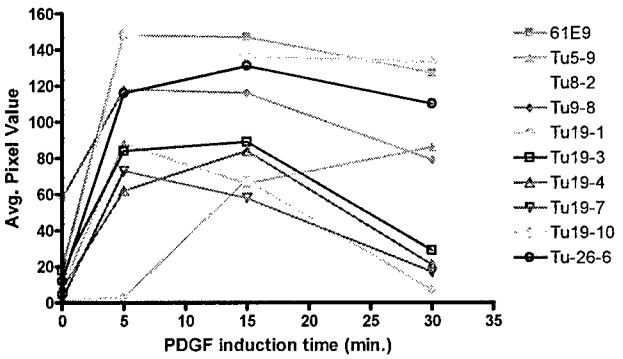
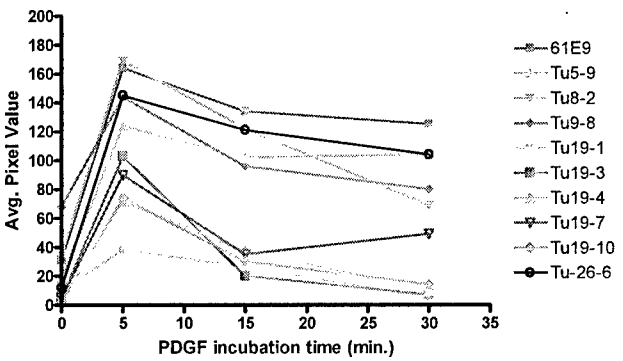


Figure 5C.
Comparison of Phospho-Erk1 Levels in Tumor Cells Grown in PDGF (10 ng/ml)



KEY RESEARCH ACCOMPLISHMENTS

- Completed comparisons of Nf1-/-, +/-, and +/+ neural crest cell (trigeminal ganglion, first branchial arch) invasiveness through fibronectin and laminin
- Completed comparisons of cranial vs. trunk mesenchymal cell invasiveness, in the context of neurofibromin deficiency, for E10, E11, and E12 populations
- Assessed the effects of PI3 kinase and MAP kinase inhibitors on Nf1-/- NCC invasiveness
- Completed Western blot time course analyses of signaling pathways activated by TGF-beta and PDGF-BB in MPNST-like sarcoma cell lines isolated from cisNf1+/-;p53+/- mice
- Initiated experiments to characterize PDGF signaling as a modifier of sarcoma cell invasiveness
- Expanded cisNf1+/-;p53+/- mouse model to include analyses of genomic instability and modifiers of tumor growth in the context of NF1-associated malignancies

REPORTABLE OUTCOMES

Manuscripts and Abstracts

- June 2003: Molecular Biology of NF1 and NF2 Meeting, oral presentation. "Mutant frequencies in NF1 tumors and Nf1+/-;Trp53+/- tissues"
- June 2003: Molecular Biology of NF1 and NF2 Meeting, poster presentation. "Invasiveness of neurofibromin-deficient cranial mesenchymal cells"
- June 2005: Children's Tumor Foundation, Molecular Biology of NF1, NF2, and Schwannomatosis Meeting, poster presentation. "A mild mutator phenotype arises in NF1-associated malignancies"
- Miller, S.J., Li, H., Rizvi, T.A., Huang, Y., Johansson, G., Bowersock, J., Sidani, A., Vitullo, J., **Vogel, K.S.**, Parysek, L.M., DeClue, J.E., and Ratner, N. (2003) Brain lipid binding protein in axon-Schwann cell interactions and peripheral nerve tumorigenesis. Molecular and Cellular Biology 23, 2213-2224
- Ling, B.C., Wu, J., Miller, S.J., Monk, K.R., Shamekh, R., Rizvi, T.A., Decourten-Myers, G., **Vogel, K.S.**, DeClue, J.E., Ratner, N. (2005) Role for the epidermal growth factor receptor in neurofibromatosis-related peripheral nerve tumorigenesis. Cancer Cell 7, 65-75
- Garza, R., Hudson, R.W., Walter, C.A., and **Vogel, K.S.** (submitted) A mild mutator phenotype arises in malignancies associated with neurofibromatosis type 1. (*Cancer Research*)
- White, C., and **Vogel, K.S.** (submitted) Increased invasiveness of neurofibromin-deficient branchial arch mesenchymal and trigeminal ganglion neural crest cells. (*Developmental Dynamics*)

Cell Lines and Animal Models

- cisNf1+/-;p53+/-; lacI+ mice and sarcoma cell lines, for quantitation of mutant frequency and characterization of genomic instability
- cisNf1+/-;p53+/-;Mrg15+/- mice and sarcoma cell lines, for identification of potential modifiers of tumor latency and growth

Funding Obtained and Pending

- San Antonio Area Foundation: "Mutant Frequency in a Mouse Tumor Model for NF1" $11, 815
- San Antonio Cancer Institute: " Nf1 and Mrg15 Regulation of Tumorigenesis and Neural Cell Proliferation" $15,000
- Pending, DoD NFRP: "Nf1 Expression: Computational Analyses and Experimental Verification of Putative Cis-Regulatory Sequences"
- Pending, DoD NFRP: "Variable Expressivity in NF1: Using Mouse Models to Identify Contributions of Genomic Instability"
- Pending, San Antonio Cancer Institute: "Mutation Spectrum and Base Excision Repair in NF1"

Employment and Training Opportunities

- Rene Garza, Research Assistant
- Robert Hudson III, Senior Research Assistant
- Nikita Ruparel, Graduate Student (laboratory rotation)
- Shivani Ruparel, Graduate Student (laboratory rotation)

CONCLUSIONS

First, we have demonstrated that neurofibromin deficiency alters the motility and invasiveness of cranial neural crest and mesenchymal cell populations in early embryogenesis; loss of neurofibromin does not, however, appear to alter the invasiveness of limb-derived (mesodermal) mesenchymal cells. This is likely to reflect the variable importance and expression levels of different RasGAPs in distinct cell lineages throughout development. We would like to begin to understand how Nf1 gene expression is regulated both temporally and spatially during embryonic development and differentiation, and we have submitted a proposal to address this question.

Second, we have characterized TGF-beta and PDGF-BB signaling pathways in MPNST sarcoma cell lines isolated from cisNf1+/-;p53+/- mice, and continue to characterize these growth factors as modifiers of tumor growth and invasiveness in the context of NF1. Over the next few months, we plan to complete both the invasiveness studies, and the experiments on TGF-beta induction of Jun-kinase signaling and apoptosis in these tumor cell lines. We will include comparisons of TGF-beta effects on Schwann cells isolated from Nf1-/- and wild-type mouse embryos for the latter studies.

"So what" section Over the past few years, it has become increasingly clear that stem cells persist in a variety of adult tissues, and may in fact represent a cell population that is particularly prone to malignant transformation. NF1 is primarily a disorder of the neural crest, and therefore it is of potential interest to understand the roles of neurofibromin in regulating the behavior of these cells. Our experiments address the role of neurofibromin in modulating neural crest cell responsiveness to environmental cues, in the context of motility. Phenotypic characterization of the sarcomas that arise in cisNf1+/-;p53+/- mice is consistent with a neural crest stem cell origin for murine MPNST-like tumors, i.e. a variety of differentiated traits can be expressed by a given tumor cell. Over the past several years, our investigations of invasiveness and growth factor responsiveness in these MPNST cell lines have revealed considerable heterogeneity, and this led us to begin to quantitate genomic instability in the context of NF1 malignancies. The mouse models generated and maintained during the funding period should provide ideal systems to continue to examine genomic instability, which may account for at least some of the variable expressivity characteristic of NF1.

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